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Genes encoding the PR-4 protein wheatwin are developmentally regulated in wheat grains and respond to high temperatures during grainfill

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Abstract

Sequences encoding three wheatwins, including a novel protein not identified previously, were found among expressed sequence tags (ESTs) from grain from the US bread wheat Butte 86 and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect transcripts specific for two of the wheatwins in a variety of tissues. In the absence of pathogen challenge, wheatwin transcripts were detected in embryo, endosperm, whole grain, awns and glumes as well as in leaves, stems and roots. In both whole grain and endosperm, transcripts accumulated late in development and achieved the highest levels as grain reached maximum dry weight. When high temperature regimens (37/28 °C day/night) were imposed during grain development, the timing of transcript accumulation was compressed and maximum transcript levels were significantly higher than under a moderate 24/17 °C day/night regimen. Accumulation profiles for two wheatwin proteins identified in endosperm by 2-DE/MS were consistent with transcript profiles and flour from grain produced under high temperatures had greater amounts of the two proteins than flour from grain produced under moderate temperatures. The data confirm that wheatwin genes are developmentally regulated in the grain and suggest that wheatwin plays a role in the response of the developing grain to high temperatures in addition to its protective role against fungal pathogens. Enhanced expression of wheatwin genes in grain produced under high temperature conditions may have important implications for wheat flour quality and allergenic potential.
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Keywords: Abiotic stress; Allergens; Defense proteins; Heat stress; Flour quality; Quantitative RT-PCR

1. Introduction

Wheatwins are pathogenesis-related proteins of the PR-4 family that are highly homologous to barwin from barley. Four 13.6 kDa proteins, referred to as wheatwin1–4, have been isolated from wheat flour [1–3]. The proteins contain six cysteine residues, all linked by disulfide bridges, and differ only at residues 62, 68, 78 and 88 of the mature protein. Modeled structures of the four wheatwin proteins have been constructed based on the three dimensional structure of barwin [4]. Wheatwins1 and 2 have been found to inhibit hyphal growth of a variety of fungi [2] and the four wheatwin proteins have been shown to have distinct antifungal activities against *Fusarium culmorum*, a soil-borne fungal pathogen that causes foot rot [4]. In addition, wheatwin1 has been demonstrated to possess an RNAse activity that may be part of a mechanism for inhibiting invading pathogens [5]. The characterization of cDNAs for wheatwin1 and wheatwin2 revealed that the protein is synthesized in endosperm tissue as a larger precursor containing a signal peptide [6]. Gene sequences for wheatwins1–4 have not been isolated, although genes for five other PR-4 proteins related to wheatwins were reported recently [7].

The expression of wheatwin genes has been investigated in a number of tissues, particularly in response to pathogen challenge. Caruso et al. [8] used immunoblots to show that wheatwin proteins were specifically induced in wheat seedlings challenged with *F. culmorum* and Bertini et al. [9] demonstrated that the gene for wheatwin1 was induced in coleoptiles and roots by the same pathogen. Bertini et al. [9] also showed that the wheatwin1 gene was induced by wounding in coleoptiles,

Abbreviations: 2-DE, two-dimensional gel electrophoresis; 2-DE/MS, two-dimensional gel electrophoresis/mass spectrometry; BTH, benzo(1,2,3)thiadiazole-7-carbothionic acid *S*-methyl ester; DPA, days post-anthesis; ESTs, expressed sequence tags; JA, jasmonic acid; MJ, methyl jasmonate; NPK, nitrogen–phosphorous–potassium; PR-4 proteins, pathogenesis-related-4 proteins; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; SA, salicylic acid; SAGE, serial analysis of gene expression

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roots and leaves. In addition, wheatwin transcripts were strongly induced in coleoptiles and roots by salicylic acid (SA), the SA analog benzo(1,2,3)thiadiazole-7-carbothionic acid *S*-methyl ester (BTH) and methyl jasmonate (MJ), indicating that the expression of these genes may be under the control of both SA and jasmonate signaling pathways. Desmond et al. [10] examined the expression of wheatwin in seedling stems inoculated with *Fusarium pseudograminearum*, the fungal pathogen that causes crown rot disease. Wheatwin1 and 2 genes as well as a number of genes encoding other PR-proteins were induced in both susceptible and partially resistant wheat cultivars. Wheatwin1 and 2 genes were also induced by MJ in seedling stems from both cultivars, but by BTH in only the susceptible cultivar. Responses to unfavorable environmental conditions also are mediated by complex signaling pathways and may involve the expression of some of the same genes involved in pathogen defense [13,14]. However, the potential role of wheatwin in the response to abiotic stress has not been investigated.

Although wheatwins1–4 were isolated from wheat flour, the expression of the corresponding genes has not been investigated in detail in the grain. A proteomic study using the US wheat cultivar Butte 86 noted that proteins involved in stress and defense are more prevalent late in grain development and identified two wheatwin proteins in endosperm from grain at 36 days post-anthesis (DPA) but not at 10 DPA [11]. More recently, a study using serial analysis of gene expression (SAGE) revealed an increase in the frequency of sequence tags for defense-related genes, including two wheatwins, at late stages of grain development in a Australian wheat cultivar banks [12].

In this paper, we focus on wheatwin genes expressed in developing grains from the US bread wheat Butte 86, a cultivar that has been used extensively in controlled growth experiments aimed at evaluating the interacting effects of temperature and fertilizer on grain development and flour quality [15–18]. We use quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to compare the accumulation of transcripts corresponding to two wheatwin proteins in different plant tissues and in developing grain produced under controlled environmental regimens. Low levels of transcripts accumulated in a variety of wheat tissues in the absence of pathogen challenge. However, transcripts accumulated to the highest levels in whole grain at late stages of development. Moreover, levels of transcripts increased in developing grain in response to high temperatures.

2. Materials and methods

2.1. Growth of plants and tissue collection

The US hard red spring wheat *Triticum aestivum* cv. Butte 86 was grown in a climate-controlled greenhouse under a moderate temperature regimen with a daytime maximum of 24 °C and a nighttime minimum of 17 °C. The maximum and minimum temperatures were maintained for 5 and 11 h, respectively, and were separated by 4 h periods at 21 °C as described [15]. Plants were watered with a dilute solution of Plantex 20–20–20 nitrogen–phosphorous–potassium (NPK) using an automatic drip irrigation system equipped with a fertilizer injector. At anthesis or at specific times after anthesis, some of the plants were transferred to a second greenhouse maintained at a high temperature regimen with a daytime maximum of 37 °C and a nighttime minimum of 28 °C. The maximum and minimum temperatures were maintained for 4 and 11 h, respectively, and were separated by 4 or 5 h periods at 30 °C. Fertilizer levels were adjusted at anthesis so that plants received 300 mg/day (1×), 150 mg/day (0.5×) or no (0×) NPK during grain development. Embryo tissue was harvested from 30 DPA grain and awn, glume, stem and leaf tissue was obtained during middle stages of grain development from plants grown under the 24/17 °C regimen. Developing grain and endosperm were collected at various intervals after anthesis under both temperature regimens. Roots were harvested from seedlings grown on water-saturated sand for 20 days. All tissues were frozen in liquid nitrogen and stored at −80 °C until use.

2.2. RNA preparation and qRT-PCR

Total RNA was isolated from all tissues as described previously for endosperm [19] and treated with RQ1 RNAse-free DNAse (Promega, Madison, WI). RNA was reversed transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) according to the manufacturer's directions. Amplification reactions were carried out in a volume of 25 μl containing cDNA, 0.3 μM of forward and reverse primers and SYBR Green Supermix (Biorad Laboratories, Hercules, CA) using a Biorad iCycler with an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 45 s. At the end of the PCR cycles, a melting curve was generated and analyzed by the iCycler software.

Oligonucleotide primers for wheatwin genes were based on expressed sequence tags (ESTs) from developing grains of Butte 86. Primers were selected using Beacon Designer 4.0 Software (Premier Biosoft International, Palo Alto, CA) and synthesized by Operon (Huntsville, AL). Forward primer QF32 has the sequence 5′ GGGACACCGTCTTCACCAAG 3′ and reverse primer QR32 has the sequence 5′ CGGATACATAATC-TAGTCG 3′. Forward primer QF82 has the sequence 5′ TGAAATGAAAGGGTAAGCAAGC 3′ and reverse primer QR82 has the sequence 5′ TCAACAGCATGGATCAGTCTC 3′. Primers for an 18S rRNA used as a reference were 5′ GACGGAAGGGCACCACCAG 3′ and 5′ CCAGACAAA-TCGCTCCACCAAC 3′. Amplification efficiencies for each primer pair were calculated from standard curves generated in three independent experiments using the iCycler software. Each standard curve had a minimum of five points and R-values greater than 0.99. Average amplification efficiencies for QF32/QR32 and QF82/QR82 were 98.8% and 96.2%, respectively. Amplification efficiency for 18S RNA primers was 98.3%. Sizes of amplification products were evaluated by gel electrophoresis on 4% metaphor agarose gels (VWR International, Brisbane, CA).

For quantification of transcripts, PCR reactions were carried out in triplicate using cDNA from the equivalent of 10 ng RNA from each time point. The 18S rRNA served as a reference RNA and was amplified in parallel with target genes in all experiments. The Ct value was determined for each RNA sample and primer pair by the iCycler software. Mean normalized expression and standard errors were determined for each target gene relative to the 18S reference gene using the Q-gene core module (available at http://www.gene-quantification.de/download.html#qgene). Amplification efficiencies of each primer pair were taken into account for all calculations using equation #3 of Muller et al. [20]. Mean normalized expression was plotted as a function of chronological age for each growth experiment. Transcript accumulation was assessed in five independent growth experiments. Transcript profiles for wheatwin genes were similar under the 24/17 °C regimen in two experiments in developing grain produced with either 0× or 1× NPK and in two experiments in developing endosperm produced with 0.5× NPK. Enhanced expression of wheatwin genes under the 37/28 °C treatment was observed in four independent growth experiments that varied in the application of NPK.

2.3. Protein extraction and 2-DE analysis

Endosperm was collected from developing grain and a KCl-soluble/methanol-insoluble protein fraction was isolated as described previously [21,11]. Equal amounts of protein (18 μg) from each time point were separated by 2-DE in triplicate. Gels were digitized with a calibrated scanner (Epson Expression 800, Long Beach, CA) at 300 dpi using the same setting for all gels and analyzed using a computerized image analysis system (Phoretix 2D Version 5.01, Non-Linear Dynamics Limited, Newcastle upon Tyne, UK) [11]. Normalized volumes (individual spot volume/total spot volume × 100) were determined for each spot, averaged over the three gels, and plotted against the chronological age of the grain in DPA. Similar methods were used for flour milled from grain produced under different temperature regimens.

3. Results

3.1. Wheatwin genes expressed in Butte 86 developing grain

The dbEST of the National Center for Biotechnology Information (NCBI) includes 3649 expressed sequence tags (ESTs) generated from developing grain of the bread wheat Butte 86. Eight of these ESTs contain the entire coding sequence for wheatwin proteins. Similarities among the proteins encoded by GenBank accession nos. BQ807250, BQ804430 and BQ806289 and the homologous barley protein, barwin (Swissprot accession no. P28814) are shown in Fig. 1. All of the Butte 86 sequences encode proteins that are ~15.6 kDa and are predicted to contain a 21 bp signal peptide by signal P algorithms (available at http://www.cbs.dtu.dk/services/SignalP/). BQ807250, along with three other Butte 86 ESTs, and BQ804430, along with two other Butte 86 ESTs, encode proteins identical to wheatwin1 [1] and wheatwin2 [2] reported previously. BQ806289 is a unique EST from Butte 86. Although the sequence for BQ806289 reported in the database contains an unidentified nucleotide at position 467, reprocessing of the original trace file revealed that the sequence beginning at nucleotide 461 should be CAACGGGGTCGG rather than CAACGGNGGTCGG. Phred scores for all bases in this region were greater than 30, indicating an accuracy of base calls greater than 99.9% (G. Lazo, personal communication). Without this nucleotide, BQ806289 encodes a protein that is similar to wheatwin4 in residues 62, 68, 78 and 88 of the mature protein [3], but differs from all of the previously described wheatwin proteins in residues 4 and 107 of the mature protein (Fig. 1). The asparagine at residue 4 and the valine at residue 107 of the mature protein are found in proteins encoded by several other ESTs in the database that were obtained from

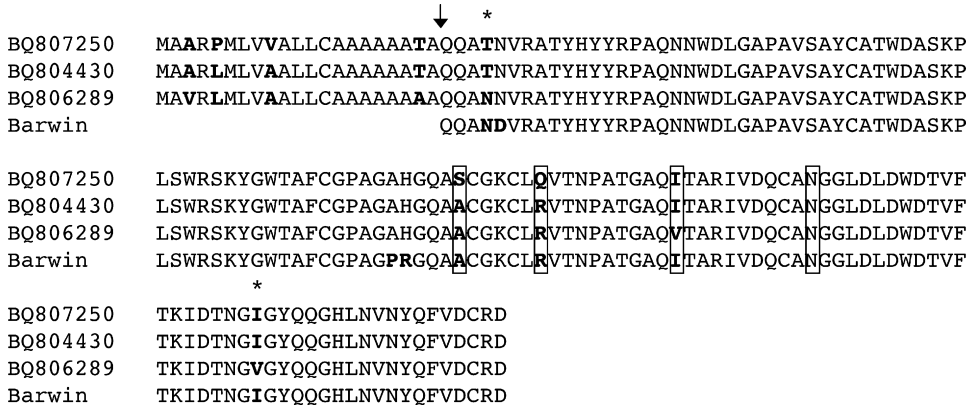

Fig. 1. Comparison of amino acid sequences of wheatwin proteins expressed in Butte 86 grain deduced from ESTs BQ807250, BQ804430 and BQ806289 and barwin, a homologous protein from barley. Amino acids that differ between the proteins are shown in bold. Putative signal peptide cleavage sites of the wheatwin proteins determined by signal P algorithms are indicated with an arrow. Boxes indicate amino acids at positions 62, 68, 78 and 88 of the mature wheatwin protein that vary among proteins reported previously [3]. Asterisk indicates changes in amino acid sequences at positions 4 and 107 of the mature wheatwin protein that were not observed in previous studies.

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QF32    BQ804430   380 GGGACACCGTCTTCACCAAG 399
        BQ807250   432 GGGACACCGTCTTCACCAAG 451
        BQ806289   433 GGGACACCGTCTTCACCAAG 452

QR32    BQ804430   468 CGACTAGATTATGTATCCG 487
        BQ807250   520 CGACTAGATTACGTTCTCC 539
        BQ806289   521 CGACTAGATGAAATGAAAG 540

QF82    BQ806289   529 TGAAATGAAAGGGTAAGCAAGC 550
        BQ807250   528 TTACGTTCTCCGTCGATCGATC 549
        BQ804430   476 TTATGTATCCGTCGATCAAAGG 497

QR82    BQ806289   603 GAGACTGATCCATGCTGTTGA 623
        BQ807250   600 GAGACTGATCCATGCTGTTAA 620
        BQ804430   547 GAGACTGGTCGATGCTGTTGA 567
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Fig. 2. Comparison of the nucleotide sequences of regions of BQ806289, BQ804430 and BQ807250 used for gene-specific primers. QF32 and QR32 match nucleotides 380–399 and 468–487 of BQ804430, respectively. QF82 and QR82 match nucleotides 529–550 and 603–623 of BQ806289, respectively. Nucleotides that differ in other wheatwin ESTs are underlined. The box indicates the positions of stop codons in the wheatwin sequences.

grain from other wheat cultivars. Barwin also has an asparagine at residue 4 of the mature protein (Fig. 1).

At the nucleotide level, the coding regions of the three wheatwins from Butte 86 are very similar. There are 9 base changes in the coding region between BQ807250 and BQ804430 that result in 2 amino acid changes in the signal peptide region and 2 in the mature protein (Fig. 1). There are 19 base changes in the coding regions of BQ807250 and BQ806289 resulting in 4 changes in the signal peptide and 5 changes in the mature protein. There are 14 base changes in the coding region between BQ804430 and BQ806289 that result in 2 amino acid changes in the signal peptide region and 3 in the mature protein. The three sequences are most different in the 3′ untranslated regions. BQ807250 and BQ804430 also differ in the 3′ untranslated regions from cDNAs AJ006098 and AJ006099 encoding wheatwin1 and 2, respectively, from the bread wheat cultivar San Pastore [6].

3.2. Primer selection for qRT-PCR

Primers were designed that were specific for two of the Butte 86 wheatwin genes. Primers QF32 and QR32 amplify a 107 bp fragment at the 3′ end of the coding region of BQ804430. Primer QF32 is a perfect match with all three wheatwin genes expressed in Butte 86 grain (Fig. 2). QR32 spans the stop codon of BQ804430 and has 14/19 identities with BQ807250 and 12/19 identities with BQ806289. The single fragment amplified by QF32/QR32 has a melting temperature of 87.5 °C and yields fragments of about 50 bp when digested with HaeIII and fragments of about 80 and 30 bp when digested with SalI that are common to all three wheatwin sequences (data not shown). Primer pair QF82/QR82 amplifies a 95 bp region of BQ806289 that has a melting temperature of 82.5 °C. QF82 is just beyond the stop codon in BQ806289 and has only 8/22 identities with both BQ807250 and BQ804430 (Fig. 2). QR82 has 20/21 identities with BQ807250 and 19/21 identities with BQ804430. The PCR product obtained with QF82/QR82 yields fragments of about 75 and 20 bp when digested with Dde I that are diagnostic of BQ806289 (data not shown).

3.3. Transcript accumulation in developing grain and other tissues

Transcript accumulation for both wheatwin genes was examined by qRT-PCR at 5-time points in developing grain produced under a 24/17 °C regimen (Fig. 3A and B). Transcripts for BQ804430 were detected at low levels at the earliest time point examined, 8 DPA, and increased as grain development progressed (Fig. 3A). Transcripts reached

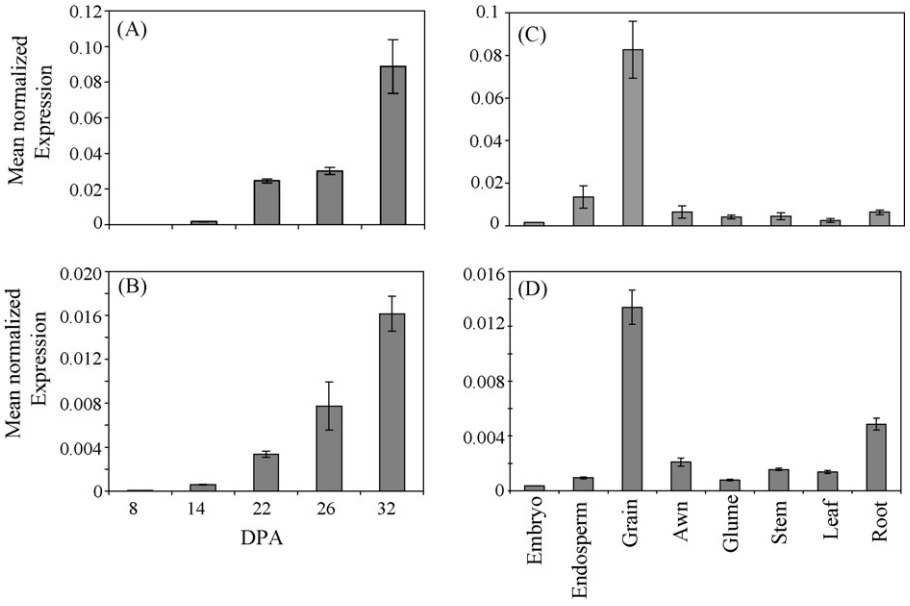

Fig. 3. Accumulation of wheatwin transcripts corresponding to BQ804430 (A and C) and BQ806289 (B and D) in developing grain between 8 and 32 DPA (A and B) and in different tissues (C and D). In panels (C) and (D), embryo, endosperm and grain tissue were harvested at 30 DPA. Transcript levels were assessed by qRT-PCR using QF32/QR32 (A and C) and QF82/QR82 (B and D). Bars indicate the standard error among triplicate reactions.

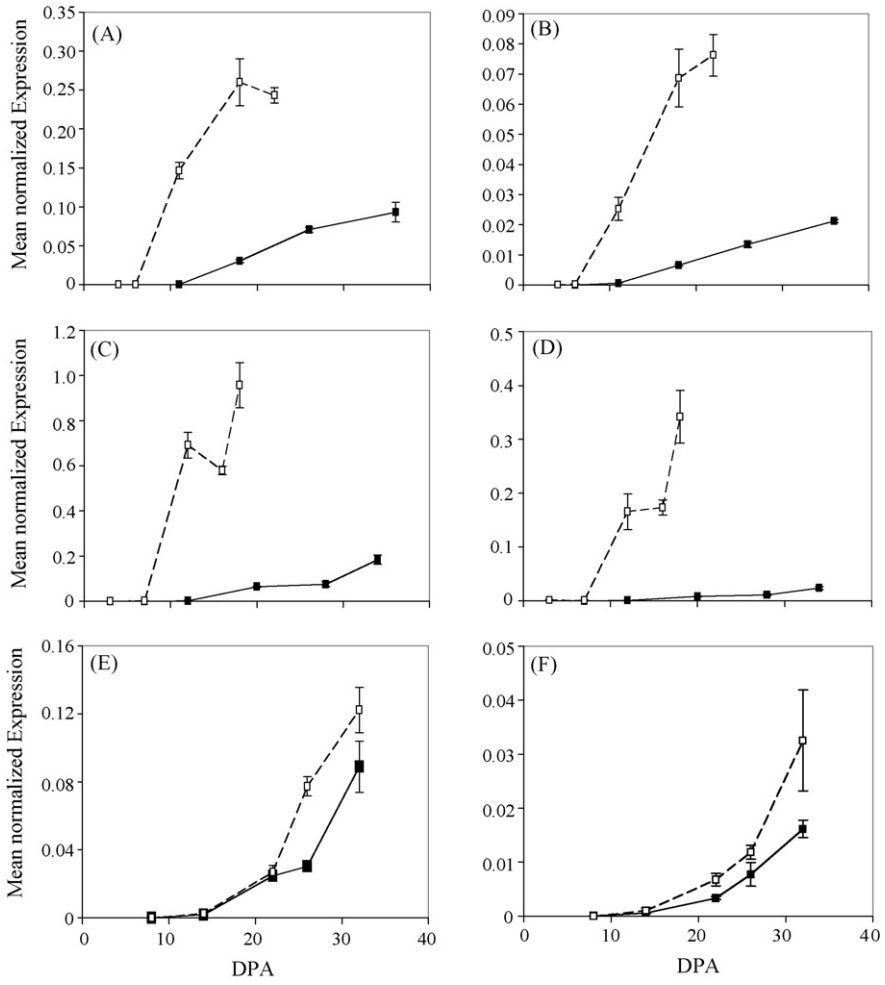

Fig. 4. Comparison of wheatwin transcript accumulation in developing grain under different temperature and NPK regimens. Panels (A–D) compare transcript accumulation under 24/17 °C (solid lines) and 37/28 °C (dashed lines) temperature regimens. Plants in (A) and (B) did not receive NPK after anthesis while plants in (C) and (D) received 1× NPK. Panels (E) and (F) compare transcript accumulation in grain produced with 1× (solid lines) or 0× NPK (dashed lines) under a 24/17 °C regimen. Transcript levels were assessed by qRT-PCR using QF32/QR32 (A, C and E) and QF82/QR82 (B, D and F). Bars indicate the standard error among triplicate reactions.

maximal levels at the last time point and were more than 1300-fold higher at 32 DPA than at 8 DPA. Transcripts for BQ806289 also were detectable by 8 DPA and showed a similar accumulation profile (Fig. 3B). As with BQ804430, transcript levels were highest at the 32 DPA time point, but these were induced only 340-fold over the 8 DPA levels.

Transcripts for the wheatwin genes also were assessed in a variety of tissues, including embryo, endosperm and whole grain, all at 30 DPA (Fig. 3C and D). Transcripts for both wheatwin genes were present at the highest levels in whole grain and were 6- and 14-fold more abundant in whole grain than in endosperm for BQ804430 and BQ806289, respectively. For comparison, transcripts for a gamma gliadin gene known to be expressed only in endosperm tissue were detected at slightly higher levels in endosperm than in whole grain in the same RNA samples (data not shown). BQ804430 transcript levels were from 13- to 32-fold higher in 30 DPA grain than in awns, glumes, stems, leaves and roots (Fig. 3C) while BQ806289 transcripts were about 3-fold higher in grain than in roots and from 6- to 17-fold higher in grain than in awns, glumes, stems and leaves (Fig. 3D). Relative to the 18S rRNA, transcripts for BQ804430 were present at higher levels than BQ806289 in most tissues. For example, in 30 DPA grain, levels of BQ804430 transcripts were about 6-fold more than BQ806289. One exception was roots where transcripts of both genes accumulated to similar levels.

3.4. Effects of high temperatures on transcript accumulation

Wheatwin transcript profiles were assessed in the grain grown under 24/17 °C and 37/28 °C regimens (Fig. 4). The time from anthesis to maturity is about 44 days under the 24/17 °C regimen and about 26 days under the 37/28 °C regimen and times to maximum water content and dry weight are notably shorter under high temperatures [15]. Under the moderate temperature regimen, wheatwin transcripts began to accumulate appreciably between 11 and 18 DPA whereas this occurred between 6 and 11 DPA under the high temperature regimen. These changes in timing were consistent with the compression

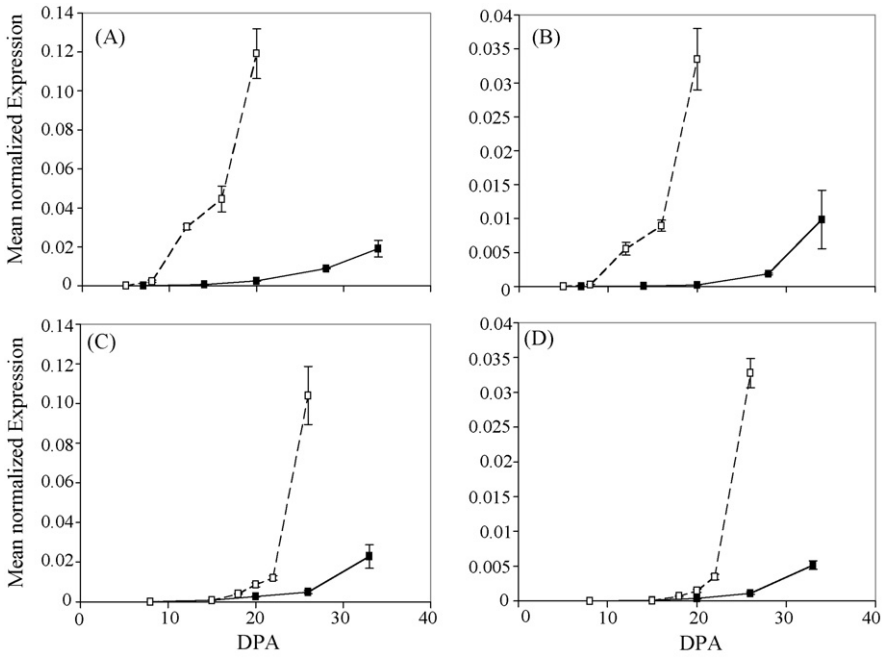

Fig. 5. Comparison of wheatwin transcript accumulation in endosperm from developing grain under moderate (24/17 °C) and high (37/28 °C) temperature regimens. In (A) and (B), the high temperature regimen was imposed from anthesis until maturity. In (C) and (D), the high temperature regimen was imposed from 15 DPA until maturity. In both experiments, plants received 0.5× NPK after anthesis. Solid and dashed lines denote transcript accumulation under the 24/17 °C and 37/28 °C regimens, respectively. Transcript levels were assessed by qRT-PCR using QF32/QR32 (A and C) and QF82/QR82 (B and D). Bars indicate the standard error among triplicate reactions.

of developmental events caused by high temperatures. In the absence of post-anthesis NPK, high temperature conditions enhanced the accumulation of wheatwin transcripts. By 11 DPA under high temperatures, transcripts were at similar or even higher levels than at 36 DPA under moderate temperatures. Late in grain development, transcript levels for BQ804430 and BQ806289 were about 2.6- and 3.6-fold higher, respectively, under the high temperature regimen (Fig. 4A and B). Expression of the two wheatwin genes also was enhanced by high temperatures when developing grain was supplied with post-anthesis NPK (Fig. 4C and D). Transcript levels for both wheatwin genes were higher by 12 DPA under high

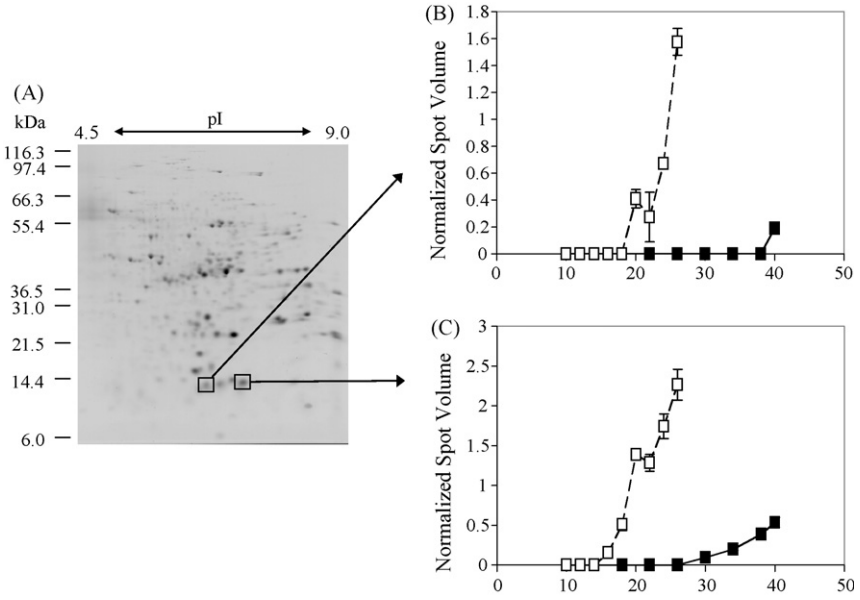

Fig. 6. Accumulation of wheatwin proteins in endosperm. Panel (A) shows a typical 2-DE pattern of KCl-soluble/methanol-insoluble endosperm proteins from 18 DPA grain produced under a 37/28 °C regimen with NPK. Proteins identified by MS as barwin or pathogenesis-related protein 4 in Vensel et al. [11] are enclosed in boxes. Panels (B) and (C) show accumulation profiles of the two proteins generated by 2-DE analyses of endosperm from grain produced under the 24/17 °C (solid lines) or 37/28 °C (dashed lines) regimens. Temperature regimens were imposed from 10 DPA to maturity and plants were supplied with 0.5× NPK. Bars indicate the standard error for each time point among triplicate gels.

temperatures than they were at 34 DPA under moderate temperatures. At late stages of grain development, transcript levels for BQ804430 and BQ806289 were about 5.2- and 14.5-fold higher, respectively, under the high temperature regimen. Maximum levels of transcripts accumulated under the moderate temperature regimen were similar in the two experiments, suggesting that NPK levels have little impact on the expression of these genes. Indeed, a direct comparison of transcript accumulation in grain produced under the 24/17 °C regimen in the presence or absence of NPK showed less than a 2-fold change in the maximum levels of BQ804430 and BQ806289 transcripts with NPK (Fig. 4C and D).

Transcript profiles also were assessed in endosperm tissue under the two temperature regimens (Fig. 5). Under the moderate temperature regimen, accumulation profiles for the two wheatwin genes were similar to those in whole grain. Transcript levels increased between 20 and 28 DPA and were at the highest levels at 34 DPA. The response to high temperature also was similar in endosperm to that observed in whole grain. By 12 DPA under the high temperature regimen, transcript levels were at nearly the same level or higher levels as at 34 DPA under the moderate temperature regimen and transcript levels at late stages of grain development were about 6.3- and 3.4-fold higher for BQ804430 and BQ806289, respectively (Fig. 5A and B).

In an additional experiment, high temperatures were applied from 15 DPA until maturity (Fig. 5C and D). While transcript levels began to increase immediately after the application of high temperatures, the most dramatic increase occurred between 22 and 26 DPA. Transcript levels of BQ804430 and BQ806289 were 4.5- and 6.4-fold higher, respectively, at 26 DPA under high temperatures than at 33 DPA under moderate temperatures.

3.5. Accumulation of wheatwin proteins in developing endosperm and flour

In a proteomic map of KCl-soluble endosperm proteins from Butte 86, peptide fragmentation data obtained by MS/MS for two protein spots yielded the highest probability matches to barwin and pathogenesis-related protein 4 [11] (Fig. 6A), both of which are very similar to proteins encoded by the wheatwin ESTs (Fig. 1). Proteins in both spots have molecular weights of ~14 kDa, but differ in p*I*. The observed isoelectric points of the two proteins were about 6.8 and 6.1. Accumulation profiles for the two proteins were examined in endosperm from Butte 86 grain produced under the 24/17 °C regimen or subjected to the 37/28 °C regimen from 10 DPA until maturity. Under the moderate temperature regimen, both proteins accumulated late in grain development. The less basic protein was first detected at 40 DPA (Fig. 6B) while the more basic protein appeared by about 30 DPA (Fig. 6C). Under the high temperature regimen, the less basic protein began to accumulate at 20 DPA and increased in amount over the duration of grain development. Levels of the protein were about eight-fold higher at the end of development under the high temperature regimen than under the moderate regimen. The more basic protein was first detected at 16 DPA and was accumulated to levels about four-fold higher by the end of development under the high temperature regimen than under the moderate regimen.

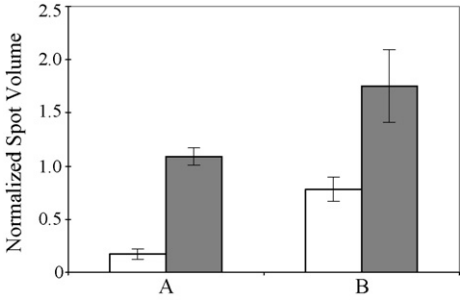

Fig. 7. Accumulation of wheatwin proteins in a KCl-soluble/methanol-insoluble protein fraction from flour prepared from grain produced under a 24/17 °C (open bars) or 37/28 °C (shaded bars) regimen with 0.5× NPK. The less basic protein shown in Fig. 6A is denoted by (A) and the more basic protein is denoted by (B). Bars indicate the standard error among triplicate gels.

Levels of both proteins also increased in white flour produced from grain subjected to the high temperature regimen (Fig. 7). White flour is derived predominantly from endosperm tissue. In flour produced from the same experiment shown in Fig. 5A and B, the less basic protein encompassed ~0.17% of a KCl-soluble/MeOH-insoluble fraction from grain produced under a 24/17 °C regimen, but 1.1% from that produced under a 37/28 °C regimen, a 6.3-fold increase with heat (Fig. 7A). The more basic protein represented ~0.8% of the protein in this fraction under the 24/17 °C regimen and ~1.8% of the protein under the 37/28 °C regimen, a 2.2-fold increase with heat (Fig. 7B).

4. Discussion

A survey of ESTs from Butte 86 developing grain identified DNA sequences encoding proteins identical to wheatwin1 and wheatwin2 as well as a novel protein containing single amino acid substitutions at two new sites. Transcripts for wheatwin2 and the novel protein were distinguished using primers that amplify the 3′ ends of the corresponding ESTs. In the absence of pathogen challenge, transcripts for both wheatwin genes were detected in awns, glumes, whole grain, embryo, and endosperm as well as in leaves, stems and roots. However, differences were revealed in the levels of transcripts in different tissues. Bertini et al. [9] also found wheatwin transcripts at very low levels in coleoptiles and roots in the absence of fungal challenge and Desmond et al. [10] demonstrated low levels of wheatwin transcripts in stems, but neither study distinguished transcripts for specific wheatwin isoforms.

In whole grain as well as in endosperm, transcripts for two wheatwin proteins were detectable at low levels early in development and increased in amount as the grain matured. However, maximum transcript levels were significantly higher in whole grain than in endosperm relative to the reference RNA, suggesting that these genes are highly expressed in bran layers or aleurone tissue. The data suggest that wheatwin proteins play roles in both the developing endosperm and other parts of the

grain. In the endosperm, it is tempting to speculate that wheatwin may be involved in the shutdown of metabolic processes since the time of maximum transcript accumulation coincides with the time that the endosperm tissue undergoes programmed cell death [15] and the protein has been shown to possess an RNase activity [5]. The greater presence of wheatwin transcripts in grain tissues other than endosperm and embryo is consistent with the notion that these proteins are involved in a protective mechanism against pathogens in outer tissue layers of the grain. The accumulation of wheatwin transcripts late in grain development is consistent with data reported by McIntosh et al. [12] using SAGE analysis that found the sequence tags CATGGAACGAAATAAAGTGGA and CATGGAATGAAATAAAGTGGA more prevalent at late stages of grain development. These tags correspond to wheatwin genes represented by BQ807250 and BQ804430, respectively.

At least two wheatwin genes also play roles in the response of the grain to high temperatures. High temperatures not only altered the timing of gene expression in a manner consistent with the compression of developmental events [16], but also increased the levels of transcripts corresponding to two wheatwin genes in both endosperm and whole grain. Two wheatwins identified by 2-DE/MS also increased proportionately in endosperm from developing grain and in the resulting flour in response to high temperatures.

Wheatwin transcripts were not induced immediately upon the application of high temperatures, but rather were accumulated at enhanced levels from about the time that grain reached maximum water content, corresponding to about 23 DPA under the 24/17 °C regimen and 11 DPA under the 37/28 °C regimen [15]. Thus, it appears that the response to temperature is superimposed upon the developmental expression of the genes. It is also interesting that increases in the levels of wheatwin transcripts in response to high temperatures generally were less than the induction observed in seedlings in response to *F. pseudograminearum*, MJ and BTH by Desmond et al. [10]. However, it is likely that the basal level of expression of wheatwin genes in seedlings is much less than in grain at late stages of development and that the grain may be in a heightened state of readiness even in the absence of applied stress.

The data provide yet another example of the connections between developmental processes and responses to both abiotic stress and pathogens and underscore the need to examine the signaling pathways that control the different responses. Cross talk among SA, JA and ethylene signaling pathways has been documented in many tissues and plants, and has been studied most extensively in *Arabidopsis* [14]. High temperatures may activate specific defense signaling pathways in the wheat grain. Recent proteomic studies have demonstrated that a number of other proteins involved in defense increase in response to high temperatures, including non-specific lipid transfer proteins [21], several seed chitinases, and a peroxidase (Hurkman et al., in preparation). It is also noteworthy that the increases in the relative proportions of proteins identified as wheatwins were some of the largest changes observed in response to high temperatures in a proteomic survey of endosperm proteins (Hurkman et al, in preparation).

Given multiple functions of wheatwins in the grain and other tissues, it might be expected that the regulation of these genes will be complex. While genomic sequences corresponding to wheatwins1–4 have not yet been isolated, genes for a number of wheat PR-4 genes that constitute a new sub-family of wheatwin-like proteins have been obtained. These genes have complex expression patterns in coleoptiles, roots and leaves in response to SA, MeJA, wounding and *F. culmorum* challenge and promoter regions from these genes contain numerous *cis*-acting elements known to be involved in stress induction [7].

Although wheat is able to withstand high temperature conditions during grain development, high temperatures have a pronounced effect on the duration of grain development and result in a significant decrease in grain size as well as changes in the functional properties of the resulting flour. Wheatwins represent a relatively small proportion of the total flour protein. Even in flour produced under high temperature conditions, these proteins encompassed only 1–2% of a fraction that represents about 11% of the total flour protein [22]. Nonetheless, it is possible that these proteins influence the quality of wheat flour. It is interesting that wheatwin was identified by MS in a dough liquor fraction prepared from white flour [23], suggesting that these proteins play a role in stabilizing gas bubbles in dough and can influence the crumb structure of bread. An additional consideration is the possibility that wheatwins may be allergens. Many food allergens are small proteins that are stable at low pH and resistant to proteolysis. About 25% of the plant proteins listed as allergens are PR-proteins, including a number of PR-4 proteins [24]. It is not known whether wheatwin reacts with IgE from patients with wheat allergies. However, wheatwin is related to hevein, a protein from *Hevea braziliensis* that is a major allergen in latex. Since one consequence of high temperatures is an increase in the levels of wheatwins in the flour, further studies are warranted on the allergenic potential of the protein and possible effects on flour quality.

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